Supplementary Material (ESI) for Chemical Communications

Electronic Supplementary Information (ESI)

Thymine, Adenine and Lipoamino Acid Based Gene Delivery Systems

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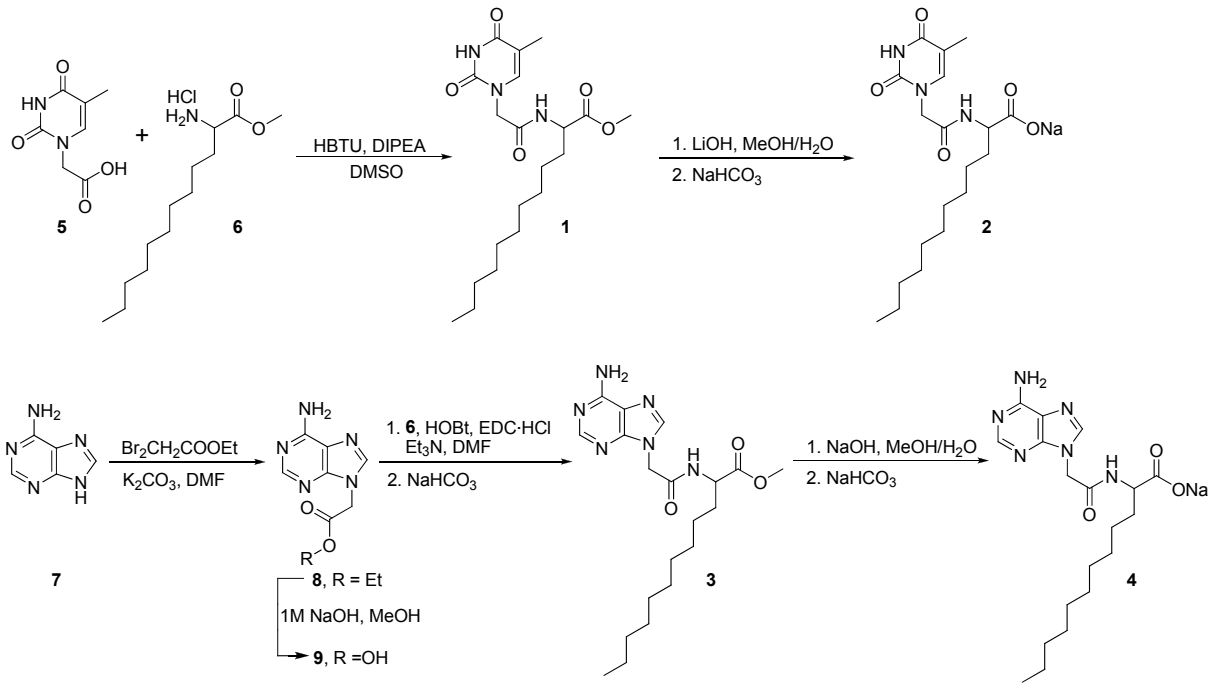
Materials and Methods

Diisopropylethylamine (DIPEA) was purchased from Auspep (Melbourne, VIC, Australia). O-Benzotriazole-*N*,*N*,*N'*,*N'*-tetramethyl-uronium-hexafluoro-phosphate (HBTU) was obtained from GL Biochem Ltd (Shanghai, China). Deuterated solvents (D_2O, CD_3OD, $CDCl_3$ and d_6-DMSO) were manufactured by Cambridge Isotope Laboratories Inc. (Andover, MA, USA). All other reagents were purchased in analytical grade or higher purity from Sigma-Aldrich (Castle Hill, NSW, Australia) or Merck Pty (Kilsyth, VIC, Australia). ^{1}H- and ^{13}C- NMR were recorded on a Bruker Avance 300, 400 or 500 spectrometer (Bruker Biospin, Rheinstetten, Germany) at 300, 400 or 500 MHz and 298 K. High-resolution mass spectrometry (HRMS) data were obtained on a Qstar Pulsar instrument (Applied Biosystems) operating in positive-ion electrospray mode. Analytical RP-HPLC was performed using Agilent 1100 series (Agilent Technologies, Inc., USA) using a 0–100% linear gradient of solvent B over 40 min with a 1 mL/min flow rate and detection at 214 nm. Solvent A consisted of 0.1% (v/v) aqueous TFA and solvent B consisted of 90% ACN/H_2O + 0.1% TFA. Separation was achieved on a Vydac (Hesperia, CA) analytical C4 column (214TP54; 5 μm; 4.6 × 250 mm).

Synthesis



Scheme S1

As depicted in Scheme 1, thymine derivative **1** was prepared from commercially available thymin-1-ylacetic acid (**5**) and methyl 2-amino-D,L-dodecanoate (**6**)[1] using HBTU/DIPEA (O-benzotriazole-*N*,*N*,*N'*,*N'*-tetramethyl-uronium-hexafluoro-phosphate/diisopropylethylamine) coupling method. The hydrolysis of **1** with lithium hydroxide, followed by work up under acidic conditions afforded compound **2** in a free acid form, which was converted to a sodium salt with sodium bicarbonate. Synthesis of adenine derivatives **3** and **4** involved alkylation of adenine (**7**) with ethyl bromoacetate. This reaction proceeded with excellent selectivity as previously reported.[2] The resulting ethyl ester **8** was hydrolyzed with sodium hydroxide to afford free acid **9**. This product once precipitated under acidic conditions was insoluble in a wide range of solvents (DMSO, acetone, water, methanol, DMF and chloroform) thus was used for the next step without any purification. To avoid possible by-product formation (reaction of carboxylic moiety with C-6 amine group of **9**) during preactivation with HBTU/DIPEA, HOBt/EDC (*N*-hydroxybenzotriazole/1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride) method was applied to afford compound **3**. Due to extremely low solubility of **9**, reaction proceeded very slowly and needed a period of three days for completion. Despite a prolonged reaction time, acylation of the free adenine NH_2 group was not detected and finally compound **3** was afforded with reasonable yield. The hydrolysis of **3** with sodium hydroxide, followed by work up under acidic conditions afforded compound **4** in a free acid form, which was converted to a sodium salt with sodium bicarbonate.

_N_-(thymin-1-ylacetyl)-2-amino-D,L-dodecanoic acid methyl ester, 1

Thymin-1-ylacetic acid (**5**) (0.18 g, 0.98 mmol), diisopropylethylamine (0.33 mL, 1.9 mmol) and 0.5 M O-benzotriazole-*N*,*N*,*N'*,*N'*-tetramethyl-uronium-hexafluoro-phosphate in dimethylformamide (1.8 mL, 0.90 mmol) were suspended in dimethyl sulfoxide (10 mL), and 2-amino-D,L-dodecanoic acid methyl ester (**6**) (0.20 g, 0.75 mmol) was added. The resulting solution was stirred overnight at room temperature, diluted with ethyl acetate (200 mL) and washed successively with 2.5% hydrochloric acid (2 x 50 mL), water (50 mL), diluted aqueous sodium bicarbonate (2 x 50 mL), water (50 mL) and brine (50 mL). The organic phase was dried over magnesium sulfate, and evaporated under reduced pressure, redissolved in ethyl acetate and washed once more with aqueous sodium bicarbonate and brine, dried over magnesium sulfate, and evaporated under reduced pressure, to give compound **1** without further purification after lyophilisation from acetonitrile (0.28 g, 95 %) as a white powder.

(Found: C, 60.65; H, 8.5; N, 10.2. Calc. for $C_{20}H_{33}N_3O_5$: C, 60.7; H, 8.4; N, 10.6); δ_H(300 MHz; DMSO-d6); 8.55 (d, *J* = 7.5 Hz, 1 H, N*H*CH), 7.40 (d, *J* = 1.2 Hz, 1 H, NC*H*CCH$_3$), 4.36, 4.29 (2d, *J* = 16.8 Hz, 2 H, NC*H*$_2$CO), 4.23 (dt, *J* = 7.8, 5.6 Hz, 1 H, NHC*H*CH$_2$), 3.61 (s, 3 H, OC*H*$_3$), 1.73 (d, *J* = 1.0 Hz, 3 H, NCHCC*H*$_3$), 1.7-1.5 (m, 2 H, NHCH*CH*$_2$), 1.23 (bs, 16 H, (C*H*$_2$)$_8$CH$_3$), 0.84 (t, *J* = 6.7 Hz, 3 H, (CH$_2$)$_8$C*H*$_3$); δ_H(100 MHz; DMSO-d6) 172.4, 167.1, 164.4, 150.9, 142.4, 107.9, 52.0, 51.9, 48.9, 31.3, 31.1, 29.0, 28.9, 28.8, 28.7, 28.5, 25.1, 22.1, 13.9, 11.9. HRMS (ESI-TOF): calcd for $C_{20}H_{33}N_3O_5Na$ 418.2312 (M^+ + Na), Found 418.2316. Purity was 99 % (HPLC analysis at 214 nm).

_N_-(thymin-1-ylacetyl)-2-amino-D,L-dodecanoic acid sodium salt, 2

Compound **1** (0.22 g, 0.56 mmol) was suspended in methanol (27 mL) and lithium hydroxide (84 mg, 2.0 mmol) in water (9 mL) was added. The mixture was stirred for 5 hours at room temperature and then methanol was evaporated under reduced pressure. Additional water (20 mL) was added and the solution was washed with cold dichloromethane (50 mL). The pH was adjusted to 2 with 5 % hydrochloric acid and the aqueous solution was extracted with ethyl acetate (100 mL). The organic phase was washed with brine (20 mL), dried over magnesium sulfate, and evaporated under reduced pressure. The resulting solid was redissolved in water with sodium bicarbonate (37 mg, 0.44 mmol) and washed with dichloromethane to give after lyophilisation compound **2** (0.19 g, 82 %) as a sodium salt without further purification. Contents of carbon, nitrogen and hydrogen according to elemental analysis did not exactly match the desired values due to the presence of salt and hydroscopic nature of the compound. A similar observation was reported previously for related derivatives.[2] However, the C/N ratio corresponded exactly to the calculated value (Found C/N 0.181 Calc. for $C_{19}H_{30}N_3NaO_5$: C/N 0.184); δ_H(400 MHz; DMSO-d6) 7.89 (d, *J* = 7.2 Hz, 1 H, N*H*CH), 7.40 (s, 1 H, NC*H*CCH$_3$), 4.32, 4.27 (2d, *J* = 16.5 Hz, 2 H, NC*H*$_2$CO), 3.95-3.90 (m, 1 H, NHC*H*CH$_2$), 1.73 (s, 3 H, NCHCC*H*$_3$), 1.7-1.4 (m, 2 H, NHCH*CH*$_2$), 1.22 (bs, 16 H, (C*H*$_2$)$_8$CH$_3$), 0.84 (t, *J* = 7.1 Hz, 3 H, (CH$_2$)$_8$C*H*$_3$); δ_H(100 MHz; DMSO-d6) 173.8, 165.8, 164.4, 151.0, 142.5, 107.8, 53.8, 49.3, 32.4, 31.3, 29.1, 29.0, 25.1, 22.1, 13.9, 11.9. HRMS (ESI-TOF): calcd for $C_{19}H_{31}N_3O_5Na$ 404.2156 (M^+ + Na), Found 404.2161. Purity was 99 % (HPLC analysis at 214 nm).

Adenin-9-ylacetic acid ethyl ester, 8

Adenine (**7**) (1.0 g, 7.5 mmol) and potassium carbonate (1.0 g, 7.5 mmol) were suspended in dry dimethylformamide (40 mL) and ethyl bromoacetate (0.83 mL, 7.5 mmol) was added

under nitrogen atmosphere. After stirring overnight at room temperature the solvent was evaporated under reduced pressure. The remaining oil was mixed with water (100 mL) resulting in crystallization. The crystals were filtered off and washed with water and ether to give compound **8** (1.2 g, 71 %). Spectroscopic data were identical to those previously reported.[2]

***N*-(adenin-9-ylacetyl)-2-amino-D,L-dodecanoic acid methyl ester, 3**

Compound **8** (0.5 g, 2.3 mmol) was suspended in methanol (30 mL), cooled to 0 °C, and 1 M sodium hydroxide (10 mL) was added. The resulting solution was stirred for 30 minutes at 0 °C. The pH was adjusted to 2 with 5 M hydrochloric acid; the resulting precipitate was filtered off and washed with water and ethanol to give adenin-9-ylacetic acid (**9**) (0.38 g). To this compound (0.2 g, 0.87 mmol) suspended in dimethyl sulfoxide triethylamine (0.21 mL, 1.2 mmol), *N*-hydroxybenzotriazole (0.16 g, 1.0 mmol), and 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (0.2 g, 1.0 mmol) were added. The reaction mixture was stirred at room temperature for 3 days. The precipitate was filtered off and washed by ethyl acetate (300 mL). The ethyl acetate phase was washed successively with water (2 x 50 mL), diluted aqueous sodium bicarbonate (50 mL), and brine (50 mL). The organic phase was dried over magnesium sulfate, and evaporated under reduced pressure and redissolved in ethyl acetate (100 mL). 5% Hydrochloric acid (50 mL) was added to the solution resulting in precipitation. After addition of petroleum ether (50 mL), the precipitate was filtered off and washed by petroleum ether to give compound **3** as a hydrochloric acid salt. The resulting solid was suspended in water (50 mL) and sodium bicarbonate (0.15 g, 1.7 mmol) was added. After sonication and centrifuging the aqueous phase was decanted. The process was repeated two times with water instead of sodium bicarbonate to give compound **3** (0.30 g, 62 % based on **8**) without further purification after lyophilisation from acetonitrile/water as a white powder. (Found C/N 0.349 Calc. for $C_{20}H_{32}N_6O_3$: C/N 0.350); δ_H(400 MHz; DMSO-d6) 8.74 (d, J = 7.6 Hz, 1 H, N*H*CH), 8.09 (s, 1 H, C_2H), 8.04 (s, 1 H, C_8H), 7.20 (s, 2 H, NH_2), 4.89 (s, 2 H, NCH_2CO), 4.24 (dt, J = 8.5, 5.5 Hz, 1 H, NHC*H*CH_2), 3.61 (s, 3 H, OCH_3), 1.71-1.56 (m, 2 H, NHCHCH_2), 1.23 (bs, 16 H, $(CH_2)_8CH_3$), 0.85 (t, J = 7.1 Hz, 3 H, $(CH_2)_8CH_3$); δ_H(100 MHz; DMSO-d6) 172.3, 166.6, 155.8, 152.2, 149.7, 141.7, 118.2, 52.0, 51.9, 44.6, 31.3, 31.0, 28.94, 28.89, 28.8, 28.7, 28.5, 25.1, 22.1, 13.9. HRMS (ESI-TOF): calcd for $C_{20}H_{33}N_6O_3$ 405.2609 (M^+ + H), Found 405.2613. Purity was 99 % (HPLC analysis at 214 nm).

_N_-(adenin-9-ylacetyl)-2-amino-D,L-dodecanoic acid sodium salt, 4

Compound **3** (0.21 g, 0.51 mmol) was suspended in methanol (20 mL) and sodium hydroxide (82 mg, 2.0 mmol) in water (10 mL) was added. The mixture was stirred for 4 hours at room temperature and then methanol was evaporated under reduced pressure. Additional water (20 mL) was added and the pH was adjusted to 2 with 5 M hydrochloric acid resulting in precipitation. The precipitate was filtered off and washed by water to give compound **4** as a hydrochloric acid salt. The resulting solid was suspended in water (50 mL) and sodium bicarbonate (0.13 mg, 1.5 mmol) was added. After sonication and centrifuging the water phase was decanted. The process was repeated two times with water instead of sodium bicarbonate to give compound **4** (0.28 g, 45 %) without further purification after lyophilisation from acetonitrile/water as a white powder. Low yield resulted due to partial solubility of the compound in the water during the salt exchange process. (Found C/N 0.361 Calc. for $C_{19}H_{29}N_6NaO_3$: C/N 0.368); δ_H(300 MHz; DMSO-d6) 8.61 (d, J = 7.8 Hz, 1 H, N*H*CH), 8.08 (s, 1 H, C_2H), 8.04 (s, 1 H, C_8H), 7.17 (s, 2 H, NH_2), 4.88 (s, 2 H, NCH_2CO), 4.22-4.15 (m, 1 H, $NHCHCH_2$), 1.73-1.53 (m, 2 H, $NHCHCH_2$), 1.23 (bs, 16 H, $(CH_2)_8CH_3$), 0.84 (t, J = 6.9 Hz, 3 H, $(CH_2)_8CH_3$); δ_H(100 MHz; DMSO-d6) 173.3, 166.4, 155.9, 152.3, 149.7, 141.7, 118.2, 52.0, 44.7, 31.3, 31.2, 29.0, 28.9, 28.8, 28.7, 28.6, 25.2, 22.1, 13.9. HRMS (ESI-TOF): calcd for $C_{19}H_{31}N_6O_3$ 391.2452 (M^+ + H), Found 391.2456. Purity was 94 % (HPLC analysis at 214 nm).

NMR experiments

$CDCl_3$ samples were prepared by dissolving compounds **1**, **3**, and mixture of both of them, in 0.6 mL of $CDCl_3$ to get a final concentration of 1 mM for each sample. A ^1H NMR study was performed by using an Avance500 Bruker NMR spectrometer operating at 500.13 MHz for ^1H observation in $CDCl_3$ at 298K. NMR-diffusion measurements were performed by using the same 500 MHz Bruker spectrometer equipped with a gradient strength (defined by *gpz*6 and the system was set up with a gradient calibration value of 5.35G/mm) capable of producing magnetic-field-pulse gradients in the *z*-direction of 53.5 Gcm^{-1}. Experiments were performed by using the diff-4.5 pulse program with trap-shape pulsed gradients, of 4-ms duration (small delta), incremented from 0 to 36 Gcm^{-1} in sixteen steps, and the pulse-gradient separation Δ was 45 ms. ^1H NMR as well as all diffusion measurements were repeated at least three times and the reported chemical shifts/diffusion coefficients (Ds) are the mean ± standard deviation of three experiments, and all data acquisitions and processing was performed using standard Bruker Topspin 2.1 software.

D_2O and CD_3OD (both containing 10% of H_2O) samples were prepared by dissolving compounds **2** or **4** in 0.60 mL of D_2O or CD_3OD to get a final concentration of 1mM for each sample. Then 0.0606 mL of H_2O (for experiments with **2** or **4** alone) or 0.0606 mL of an aquatic solution of DNA (for experiments with DNA; it was equivalent of 0.60 μmole of adenine/thymine in DNA to get a final ratio of 1:1) was added. For these samples dissolved in aqueous solvents, stebpgp1s19 was chosen (STE bipolar gradient pulse pair, 1 spoil gradient, 3-9-19 WATERGATE solvent suppression), and measurements were carried out to suppress water peak in order to minimize possible baseline distortions near 4.8 ppm and to improve the reliability of the diffusion coefficient measurements for nearby peaks. Pulse-gradient separation Δ was 100 ms and 80, small delta was 2 ms and 3 ms for **2** and **4**, respectively.

ITC experiments

The thermodynamic interactions were calculated using a MicroCal VP-ITC Microcalorimeter (Northampton, MA, USA) with Origin 5.0 software and VPViewer 2000. DNA, single stranded from salmon testes (D 9156) was purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). DNA (133.33 ug, 12.12 ul) was taken from the stock (11 mg/mL) and water was added to 2 mL to prepare the DNA solution for the sample cell. The sample cell (1.5 mL) contained either single-stranded DNA (0.06 mM) from salmon testes or adenine (0.06 mM). The reference cell (1.5 mL) contained water and the micro-syringe contained compound **2** (1.2 mM). Experiments were performed multiple times at 25°C where compound **2** was titrated into the sample cell (10 µl every 4 min).

The thermodynamic interaction between compound **2** and single-stranded DNA or adenine was determined by isothermal titration calorimetry (Fig. S1 and S2). The top panel of Fig. S1 and S2 represents the power change to the sample cell upon the addition of compound **2** to single stranded DNA or adenine. The positive peaks indicate that the interaction is endothermic, while the following peaks are heats of dilution. The bottom panel represents the integrated area of the peaks from the top panel which is then plotted against the molar ratio. The data was corrected by subtracting the heat of dilution, which is the heat generated by the titration of compound **2** into water (data not shown). The curve was fit to the data points using a one set of sites binding model and from this the thermodynamic parameters were calculated (Table 3, main text). The concentration of DNA used in the calculations is the concentration of the base adenine within the DNA. Given 100 µg of DNA in 1.5 mL of water was placed in the sample cell and that the percentage of A-T within in the DNA is 58.8 %, the concentration of adenine in the sample cell is 0.06 mM (90 nmoles). The ratio of compound **2** required to reach equilibrium with single stranded DNA was 1.7:1. However, the ratio of compound **2** required to reach equilibrium with single stranded DNA was 0.9:1. The other main differences between the two interactions are that compound **2**/adenine has a lower binding affinity, enthalpy, entropy and Gibbs free energy.

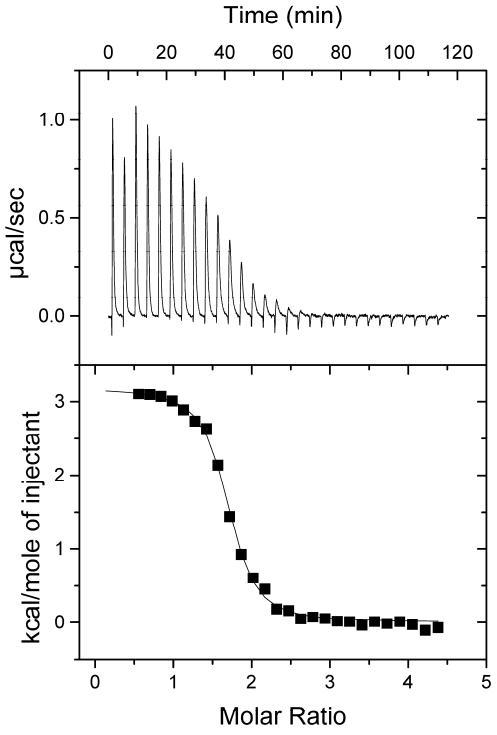


Fig. S1 Titration of compound **2** into single stranded DNA.

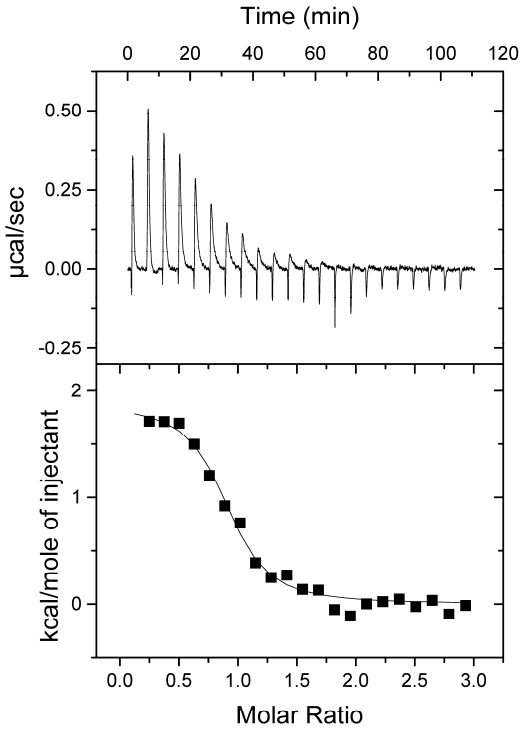


Fig. S2. Titration of compound **2** into adenine

TEM experiments

Compound **2** (0.36 mM) was prepared in water, PBS (pH 7.4) and 20 mM Hepes buffer (pH 7.2). Compound **2** / DNA complex (2:1) was prepared by adding compound **2** (90 nmoles, 0.25 mL) to DNA (45 nmoles, 0.25 mL). The sample was vortexed and allowed to stand for 30 min prior to measurement. A drop of compound **2** or **2**/DNA complex solution was allowed to air-dry onto a formvar-carbon coated 200 mesh copper grid, and the sample was stained with 2 % of uranyl acetate for 2 minutes. The sample was then characterized on a JEOL-1010 microscope operating at an accelerating voltage of 100 kV. The sample images were taken and analyzed using the AnalySIS® software (Soft Imaging Systems, Megaview III, Munster, Germany)

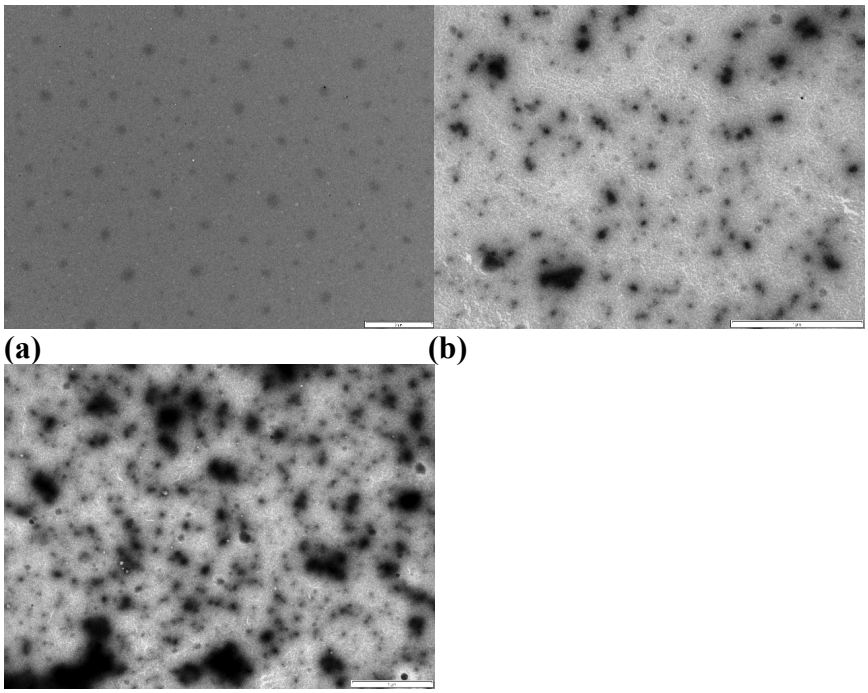
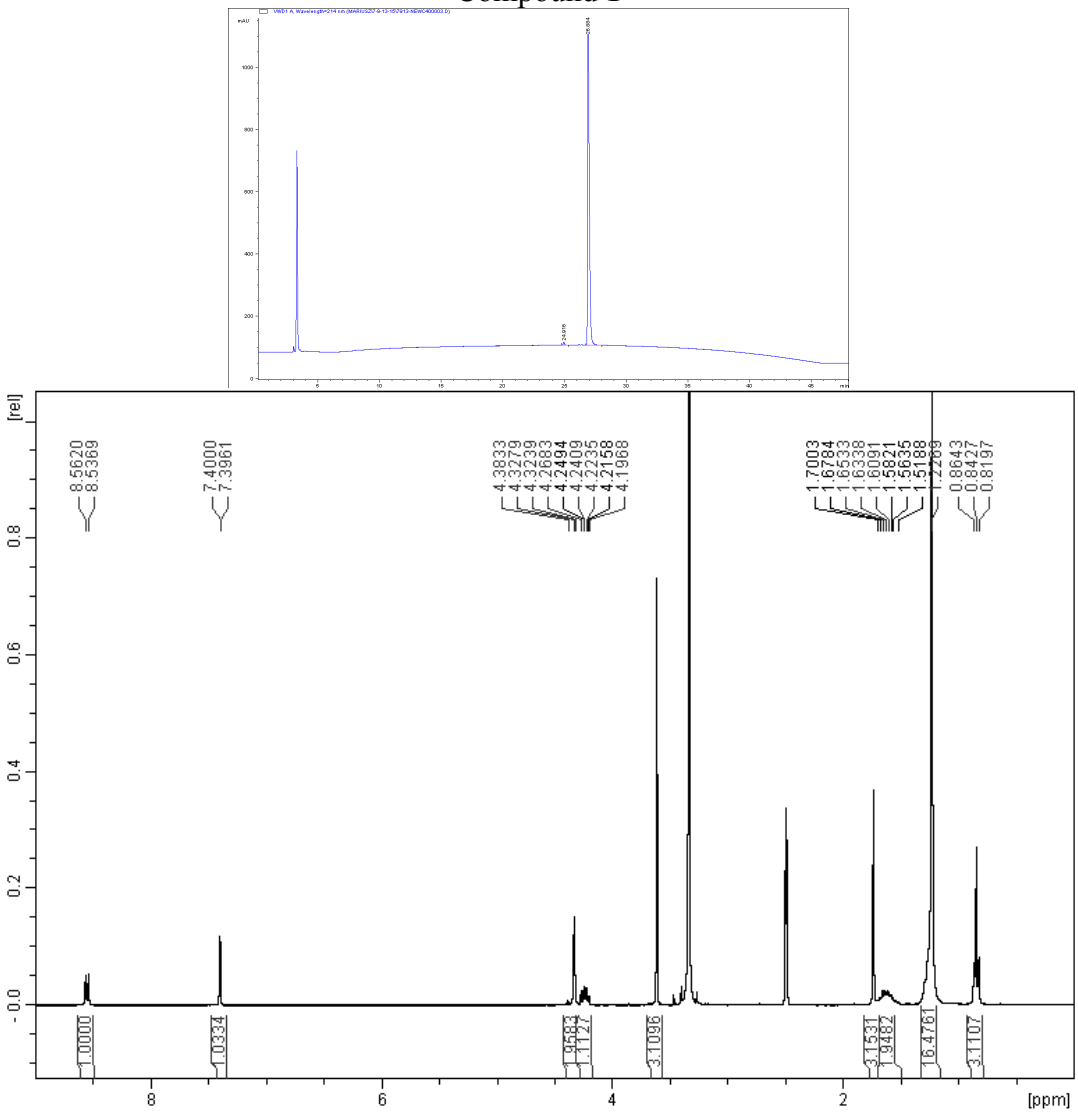
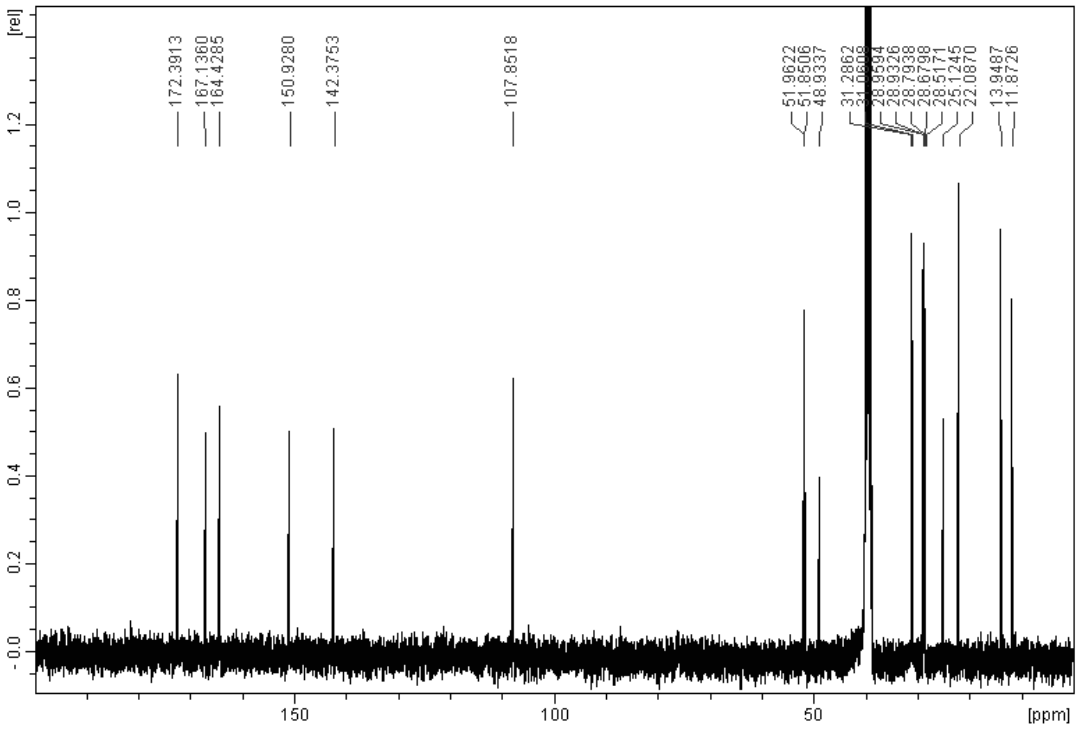


Fig. S3 Transmission electron microscopy photographs of (a) **2** in water (bar 2 μm), (b) **2**/DNA complex in water (bar 1 μm) and (c) **2**/DNA complex in PBS buffer (bar 1 μm).

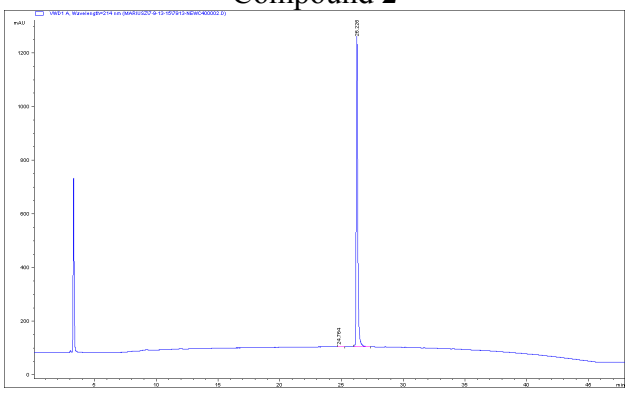
Traces of HPLC and NMR spectra for final compounds **1**-**4**.

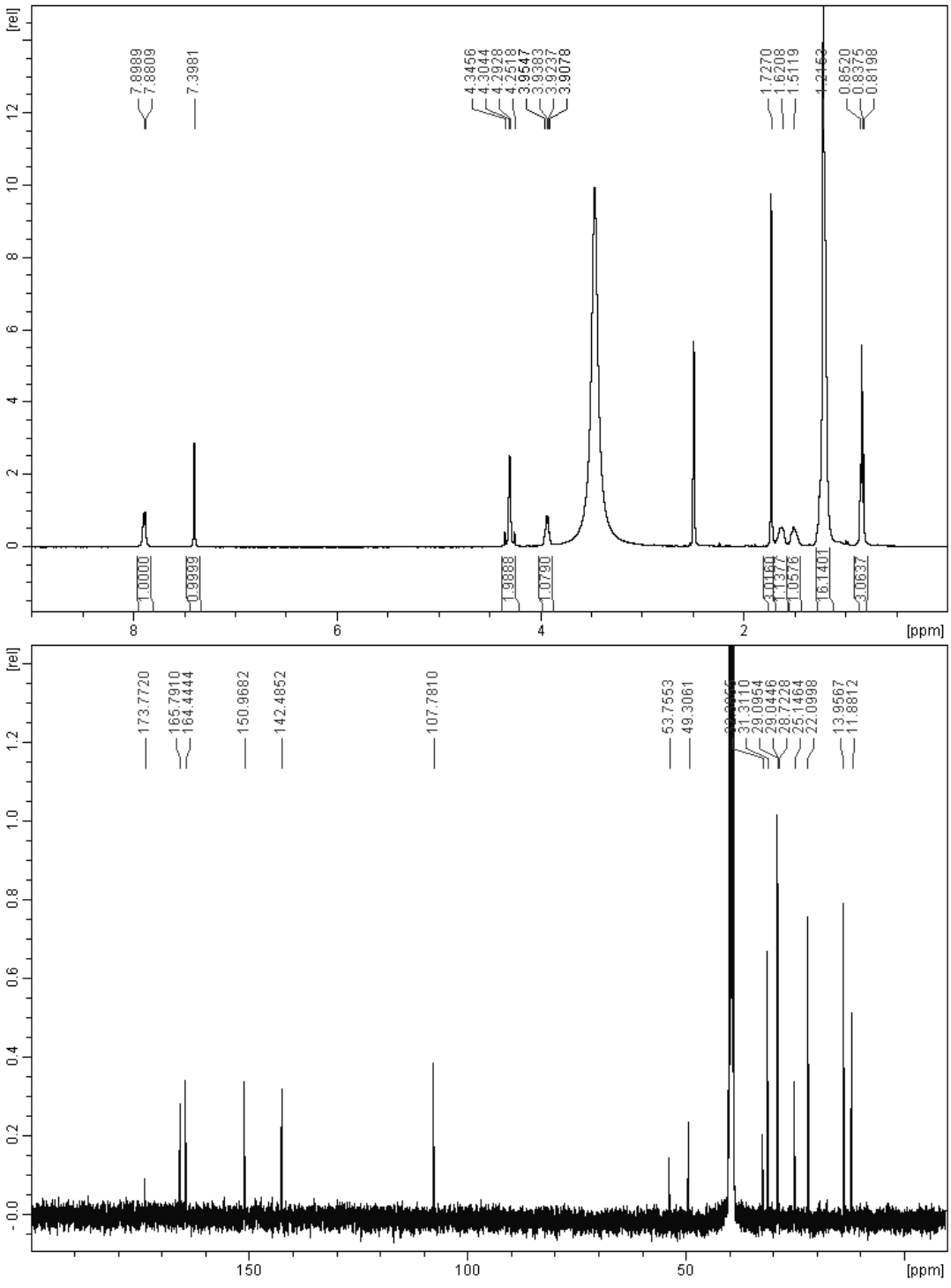
Compound **1**





Compound **2**





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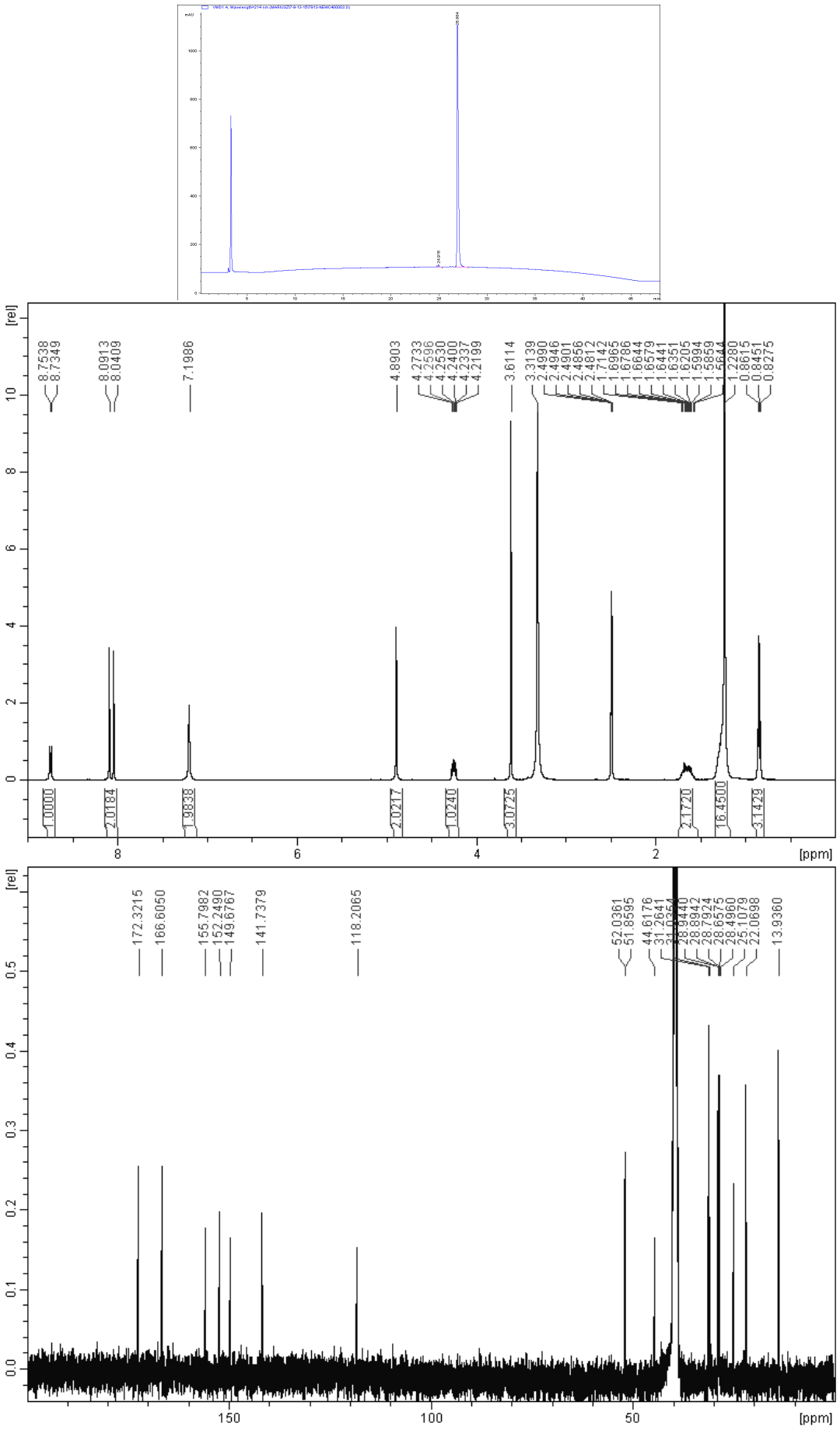
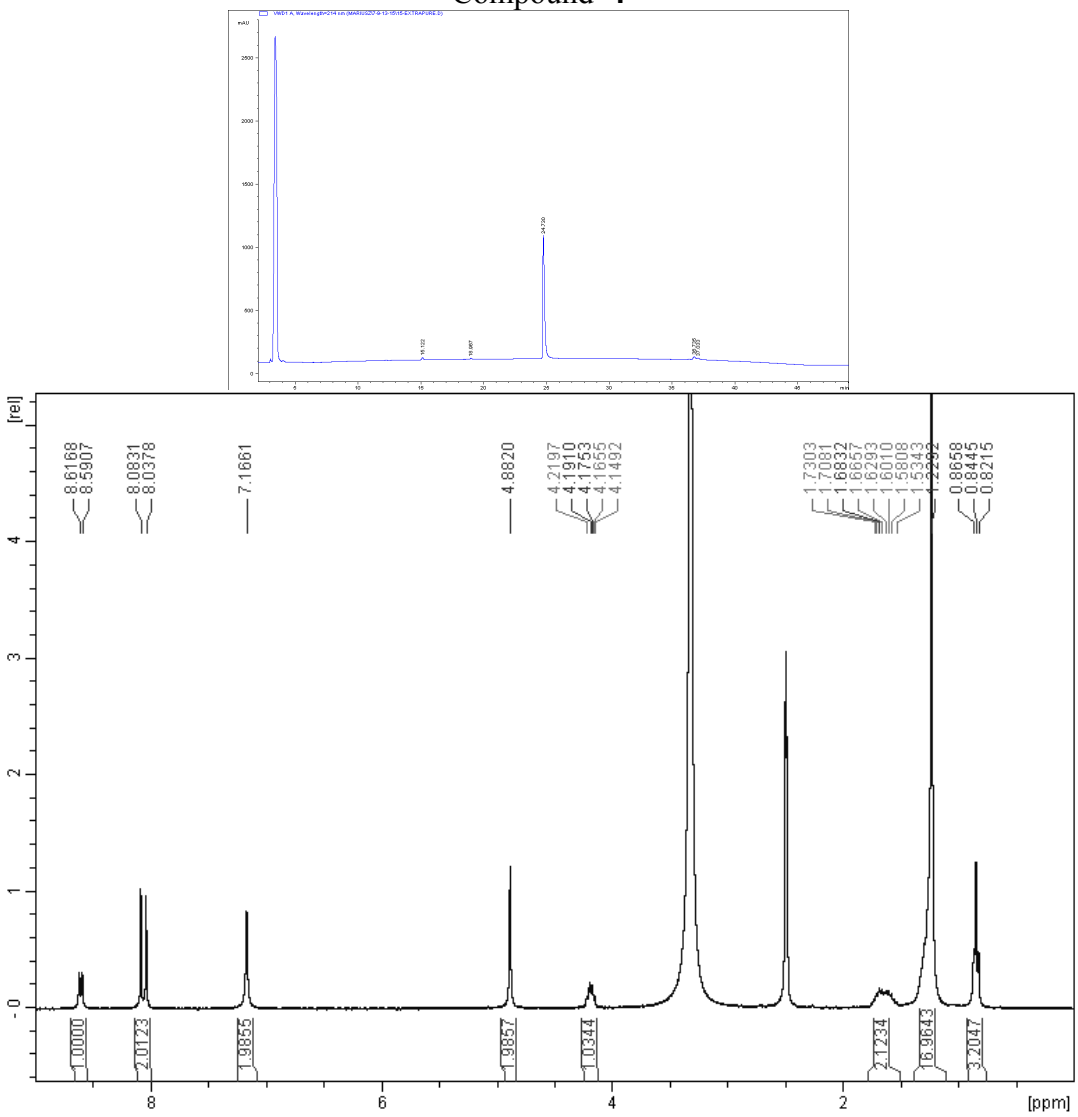
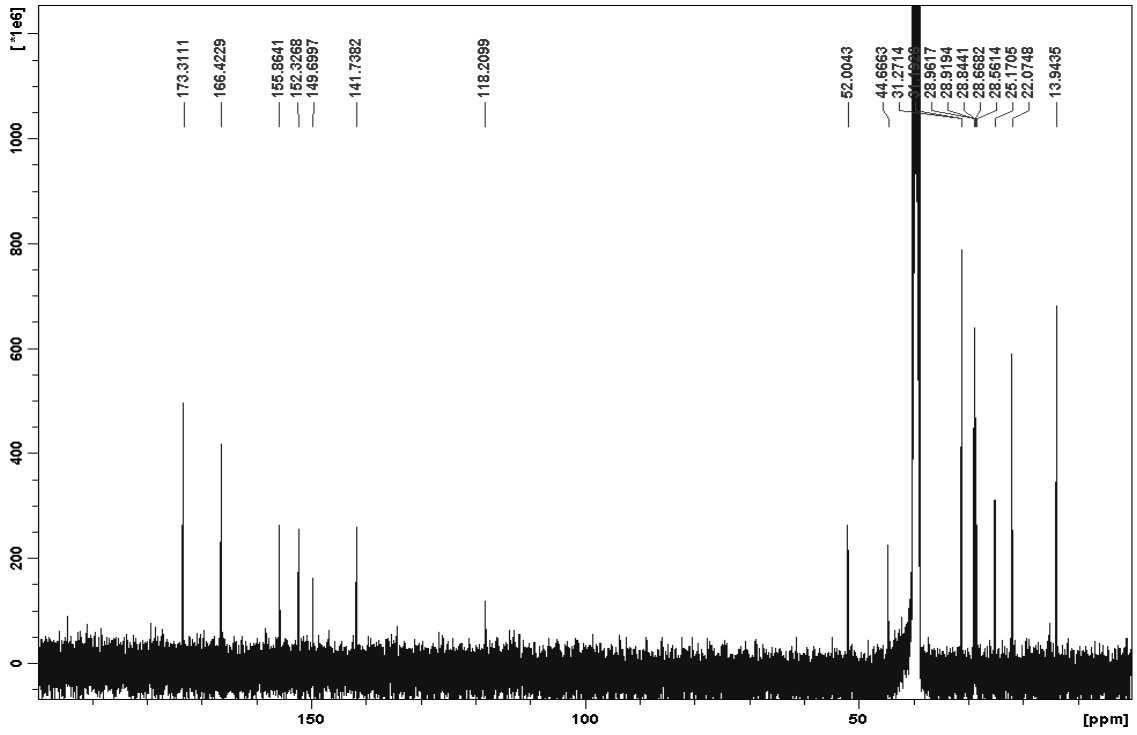


Compound **4**





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